

## DNA Vaccination of the American Crow (*Corvus brachyrhynchos*) Provides Partial Protection Against Lethal Challenge with West Nile Virus

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**SUMMARY.** The New York 1999 strain of West Nile virus (WNV) is nearly 100% fatal in the American crow (*Corvus brachyrhynchos*). We evaluated four WNV vaccine formulations in American crows, including intramuscular (i.m.) DNA vaccine, i.m. DNA vaccine with adjuvant, orally administered microencapsulated DNA vaccine, and i.m. killed vaccine. Neutralizing antibodies developed in approximately 80% of crows that received the DNA vaccine i.m. (with or without adjuvant), and in 44% that received the killed vaccine. However, no crows that received the oral microencapsulated DNA vaccine or the placebo developed WNV antibodies. All crows were challenged 10 wk after initial vaccination. No unvaccinated crows survived challenge, and survival rates were 44% (i.m. DNA vaccine), 60% (i.m. DNA vaccine with adjuvant), 0% (oral microencapsulated DNA vaccine), and 11% (killed vaccine). Peak viremia titers in the birds that survived were significantly lower as compared to titers in birds that died. Parenteral administration of a WNV DNA vaccine was associated with reduced mortality but did not provide sterile immunity.

**RESUMEN.** La vacunación del cuervo Americano (*Corvus brachyrhynchos*) con vacuna de ADN proporciona protección parcial contra el desafío letal con el virus del Oeste del Nilo.

La cepa del virus del Oeste del Nilo aislada en Nueva York en el año 1999 es casi 100% fatal en el cuervo Americano (*Corvus brachyrhynchos*). Evaluamos cuatro formulaciones de vacunas en cuervos Americanos, incluyendo una vacuna de ADN, una vacuna de ADN con adyuvante, ambas aplicadas por la vía intramuscular, una vacuna de ADN microencapsulada aplicada por la vía oral, y una vacuna inactivada aplicada por la vía intramuscular. Los anticuerpos neutralizantes se desarrollaron en aproximadamente el 80% de los cuervos que recibieron la vacuna de ADN por vía intramuscular, con o sin adyuvante, y en el 44% de los que recibieron la vacuna inactivada. Sin embargo, no se desarrollaron anticuerpos en los cuervos que recibieron la vacuna de ADN microencapsulada o en los controles no vacunados. Todos los cuervos fueron desafiados 10 semanas después de la vacunación inicial. Ninguno de los cuervos no vacunados sobrevivieron el desafío, y los porcentajes de sobrevivencia fueron del 44% para la vacuna de ADN, 60% para la misma vacuna con adyuvante, 0% para la vacuna de ADN microencapsulada, y 60% para la vacuna inactivada. Los títulos máximos de viremia en las aves que sobrevivieron fueron significativamente más bajos comparados con los títulos de los cuervos que murieron. La administración parenteral de la vacuna de ADN del virus del Oeste del Nilo estuvo asociada con una reducción de la mortalidad pero no proporcionó una inmunidad total.

**Key words:** West Nile virus, American crow, DNA vaccine, killed vaccine, oral vaccine

**Abbreviations:** ABSL-3 = animal biosafety level-3; BA1 = bovine albumin-1; E = envelope protein; i.m. = intramuscular; NY99 = New York 1999 strain; PBS = phosphate-buffered saline; PFU = plaque forming units; prM = transmembrane protein; PRNT = plaque-reduction neutralization test; SLEV = Saint Louis encephalitis virus; WNV = West Nile virus

In North America, West Nile virus (WNV; genus *Flavivirus*, family *Flaviviridae*) has caused a series of outbreaks since its discovery in 1999, resulting in thousands of human neurologic disease cases between 1999 and 2006, thousands of cases of equine encephalitis, and millions of deaths of native and exotic birds (14,17). The American crow (*Corvus brachyrhynchos*) is particularly susceptible to severe WNV infection, suffering nearly 100% mortality after experimental infection (23). Large population reductions have been documented for this species in several locations in the eastern United States, and local extirpations have been reported anecdotally after outbreaks throughout the continent

(4,5,15,29). Crows appear to be more susceptible to fatal infections caused by the New York 1999 strain (NY99) of WNV than from other closely related strains identified in Africa and Australia, indicating that a viral factor associated with NY99 is in part responsible for its virulence in crows (3). The WNV structural proteins include a capsid protein, a transmembrane protein (prM), and an envelope protein (E), all of which have been experimentally used within DNA vaccine constructs to stimulate WNV immunity (10,11,28).

Vaccination has been proposed as a means of protecting humans and horses from severe WNV infection (8,24). Progress on several potential human vaccines has been reported (12,24), and four equine vaccines are currently licensed. At present, no effective vaccine or treatment is available for birds, though trials have been conducted using a variety of formulations (2,7,9,16,20,21,22,25,26). Some

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handlers of valuable birds have opted to use equine vaccine products for their birds, in most cases with no information on safety and efficacy in birds. However, in a preliminary trial, fish crows (*Corvus ossifragus*) vaccinated with a DNA vaccine were protected, with mortality reduced from 50% to zero. Vaccination was also associated with reduction in the magnitude of viremia, suggesting that this vaccine could reduce WNV transmission from birds to mosquitoes as well as protect against disease (26). The same DNA vaccine protected mice and horses from severe WNV-associated disease (6,10), as well as provided protective immunity against natural WNV challenge in California condors (*Gymnogyps californianus*) (7).

Because birds are important amplifying hosts, one possible means for controlling WNV outbreaks would be an oral vaccine that could be utilized to vaccinate wild bird populations via feeding stations. This would be analogous to bait stations with rabies vaccine used to vaccinate wild raccoons and foxes (13). In addition, a vaccine is needed to protect valuable collections of captive birds, such as those in zoological parks and, potentially, endangered species that might be captured for vaccination, such as the whooping crane (*Grus americana*), Florida scrub jay (*Aphelocoma coerulescens*), and California condor (7). Accordingly, we further evaluated several vaccines and routes of administration for induction of protective immunity in a highly susceptible species, the American crow.

## MATERIALS AND METHODS

**Animals.** Fifty-six American crows were captured in Kansas during February and March of 2002 and transported to animal holding facilities at Colorado State University. The crows were arbitrarily separated into six groups consisting of eight to 10 birds each, identified by uniquely numbered aluminum leg bands, and placed in aviaries (1.5 × 2.4 × 1.8 m). Before vaccination, all crows were tested by plaque-reduction neutralization test (PRNT) for antibodies to WNV and St. Louis encephalitis virus (SLEV). For challenge studies, the crows were moved to an animal biosafety level-3 (ABSL-3) facility, where they were placed in stainless steel cages (0.9 × 0.9 × 0.9 m) containing two to three birds each. Handlers wore positive-air pressure respirators during the challenge studies.

Birds were provided fresh water and a mixture of dog food, cat food, and cracked corn *ad libitum*, with supplementation including fresh fruits and vegetables and vitamins. Initially, birds were provided with artificial lighting for 14 hr/day, and after moving in the ABSL-3 building, they were under a natural light cycle (via skylights) with approximately 14 hr/day of sunlight. Crows that became severely ill following WNV challenge were humanely euthanatized via intravenous overdose of sodium pentobarbital. At the end of the study, all surviving birds were euthanatized in the same manner.

**Vaccines.** The DNA vaccine used in this study was the eukaryotic expression plasmid, pCBWN (10). The transcription unit of this plasmid contains the human cytomegalovirus early gene promoter, the Japanese encephalitis virus signal sequence, the prM and E gene regions from WNV NY99 (isolate 6480), and bovine growth hormone poly(A) signal. When transfected into cultured cells, pCBWN expresses the prM and E proteins of WNV in the form of extracellular subviral particles (10). The plasmid was purified from *Escherichia coli* XL-1 blue cells (EndoFree Plasmid Giga kits, Qiagen, Valencia, CA) and resuspended in phosphate-buffered saline (PBS) buffer (pH 7.5) at a concentration of 1 µg/µl. The adjuvant used with the DNA vaccine was aluminum phosphate gel adjuvant (Alu-gel®, Cedarlane Laboratories Ltd., Hornby, Ontario, Canada), at a concentration of 1 mg/ml of vaccine in 0.2 ml of adjuvant. The oral vaccine candidate was produced by suspending a lyophilized, microencapsulated preparation of pCBWN in PBS (pH 7.5) at a concentration of 2 mg/ml as previously described (26).

A commercially available, inactivated vaccine (West Nile Innovator™, Fort Dodge Animal Health) was used as provided by the manufacturer.

**Experimental design.** After 2 wk of acclimation to their new surroundings, crows were vaccinated according to their group number. Group 1 contained nine crows injected intramuscularly (i.m.) on the breast twice at a 21-day interval with 0.2 ml of PBS containing 1 mg/ml of DNA vaccine. Group 2 contained 10 crows injected i.m. twice at a 21-day interval with the same concentration of DNA vaccine in adjuvant. Group 3 contained 10 crows that received 0.5 ml of the microencapsulated DNA vaccine orally four times at 7-day intervals. Group 4 contained nine crows injected i.m. twice at a 21-day interval with 1.0 ml of a commercially available equine killed vaccine (the same dose suggested for

horses), divided equally between two sites to reduce the volume injected per site. Two groups of unvaccinated controls were used: group 5 had eight crows injected i.m. twice at a 21-day interval with 0.2 ml of adjuvant only, and group 6 consisted of 10 crows injected i.m. twice at a 21-day interval with 0.2 ml of PBS. Crows were held in mosquito-proof rooms during all phases of the study.

During the vaccination phase, 0.6 ml of blood was collected from the jugular vein of each bird once a week and centrifuged (12,000 × *g* for 3 min); and sera from those samples were stored at -70 °C until tested for neutralizing antibodies to WNV. Nine weeks after the start of vaccinations, all of the crows were moved to an ABSL-3 facility and allowed to acclimate. One week later, all of the crows were infected subcutaneously with 0.1 ml containing 4000 plaque-forming units (PFU) of WNV strain NY99 (isolate 6625, a crow isolate with one Vero cell passage). Starting 24 hr after challenge, 0.2 ml of blood was collected via the jugular vein from each bird daily for 14 days, or until natural death or euthanatization. Blood samples were added to 0.9 ml of bovine albumin-1 diluent (BA1; consisting of M199-Hanks' salts with L-glutamine; 0.05 M TRIS-HCl, pH 7.5; 1% bovine serum albumin [Bovuminar Cohn Fraction V], pH 7.0; 0.35g/liter sodium bicarbonate; 100 units/ml of penicillin; 100 µg/ml of streptomycin; 1 µg/ml of Fungizone) with 10% fetal bovine serum, for a final serum dilution of approximately 1:10. Samples were stored at -70 °C until tested by plaque assay.

**Plaque assay.** Diluted blood samples were thawed and microcentrifuged (6000 × *g* for 10 min); 10-fold serial dilutions were then prepared in BA1 diluent. Viremia titers were determined by plaque assay in duplicate on Vero cells using the double overlay method (1), with a threshold of detection of 10<sup>1.7</sup> PFU/ml of serum. Forty-eight hours after the first agarose overlay, a second overlay containing 0.005% neutral red was added to visualize the plaques. Viral plaques were counted from each plate 24 hr after the second overlay.

**Plaque reduction neutralization test.** WNV-specific antibody titers were measured from serum samples using PRNT, as previously described (19). Briefly, 25 µl of each serum sample was added to 100 µl of BA1 diluent in a 96-well microtiter plate. A stock solution of WNV-NY99 (isolate 4132, a crow isolate with four Vero cell passages) with a viral titer of approximately 200 PFU/0.1ml was added to each serum dilution in equal volume (125 µl), resulting in a final serum dilution of 1:10 mixed with 100 PFU/0.1 ml. Mouse hyperimmune ascites fluid was used as an anti-WNV control. The serum-virus mixtures were incubated at 37 °C and 5% CO<sub>2</sub> for 1 hr and then 0.1 ml was inoculated in duplicate onto 6-well Vero cell plates. The plates were treated with the double overlay method as previously described, and the average number of plaques for each sample was compared to the average number of plaques produced by a crow-serum-free back-titration to determine the mean percentage of neutralization. For SLEV PRNT, strain TBH-28 was used.

**Statistical analyses.** Data were analyzed using STATA version 8.0. Differences in survival among the groups were measured for significance using a one-tailed Fisher's exact test. The log<sub>10</sub> mean peak viremias were compared between groups using a one-way ANOVA with Bonferroni corrections. The Student *t*-test ( $\alpha = 0.05$ ) was used to determine if differences in log<sub>10</sub> mean peak viremia titers between birds that survived and those that died were significant.

## RESULTS

All crows were seronegative for both WNV and SLEV antibodies prior to the vaccine trials. Six weeks after initial vaccination (3 wk after the second dose), only crows in groups 1, 2, and 4 demonstrated evidence of neutralizing antibodies (defined here as ≥70% neutralization at a serum dilution of 1:10) (Table 1). Neutralizing antibodies were not detected in birds that received the oral formulation of the DNA vaccine or in the birds that received adjuvant or placebo. By the ninth week after initial vaccination (6 wk after the second dose), only birds in groups 1 and 2 retained an antibody response.

None of 37 crows that failed to develop a significant antibody response by 6 wk postvaccination survived WNV challenge at 10 wk postvaccination. In contrast, 58% (11/19) of those with circulating antibodies at 6 wk survived WNV challenge 4 wk later. Although only 8% (4/48) of crows without a significant antibody response at 9 wk survived challenge 1 wk later, 88% (7/8) of crows with significant antibody levels at 9 wk survived challenge 1 wk later. Therefore, crow survival was significantly higher upon challenge for crows that had formed an antibody response by either 6 wk (one-sided Fisher's exact test,  $P < 0.001$ ) or 9 wk after vaccination (one-

Table 1. The effects of the vaccine formulation and route of administration on antibody response, viremia, and survival of American crows challenged with virulent West Nile virus.<sup>A,B</sup>

Group	Vaccine (route)	Number in group	Number (%) seropositive <sup>D</sup>		Number (%) with sterile immunity <sup>C</sup>					Number (%) survived				
					Seronegative		Seropositive		Total	Seronegative		Seropositive		Total
			6 wk	9 wk	6 wk	9 wk	6 wk	9 wk		6 wk	9 wk	6 wk	9 wk	
1	DNA (i.m.)	9	7 (78)	3 (33)	0	0	2 (29)	2 (67)	2 (22)	0 <sup>E</sup>	2 (33)	4 (57)	2 (67)	4 (44) <sup>F</sup>
2	DNA (i.m.) + adjuvant	10	8 (80)	5 (50)	0	0	2 (25)	2 (40)	2 (20)	0	1 (20)	6 (48)	5 (100)	6 (60)
3	DNA (oral)	10	0	0	0	0	NA <sup>bb</sup>	NA	0	0	0	NA	NA	0
4	Killed (i.m.)	9	4 (44)	0	0	0	0	NA	0 <sup>G</sup>	0	1 (11)	1 (25)	NA	1 (11) <sup>H</sup>
5	Adjuvant (i.m.)	8	0	0	0	0	NA	NA	0	0	0	NA	NA	0
6	PBS (i.m.)	10	0	0	0	0	NA	NA	0	0	0	NA	NA	0

<sup>A</sup>i.m. = intramuscular; NA = not applicable; PBS = phosphate-buffered saline.

<sup>B</sup>WNV challenge occurred at 10 wk postvaccination for all crows.

<sup>C</sup>Sterile immunity is defined here as lack of detectable viremia, with a detection threshold 1.7 plaque-forming units/ml serum.

<sup>D</sup>Seropositive is defined as circulating antibodies that neutralize  $\geq 70\%$  of WNV at a serum dilution of 1:10.

<sup>E</sup>Survival was significantly higher upon challenge for crows that were seropositive at 6 wk (one-sided Fisher's exact test,  $P < 0.001$ ) or 9 wk postvaccination (one-sided Fisher's exact test,  $P < 0.001$ ) *vs.* seronegative crows.

<sup>F</sup>The difference in survival between crows vaccinated parenterally with the DNA vaccine *vs.* those receiving placebo was significant (one-sided Fisher's exact test,  $P < 0.0046$ ).

<sup>G</sup>One crow with undetectable viremia levels was only able to be tested at a serum dilution of  $\geq 1:1000$ .

<sup>H</sup>The difference in survival between crows vaccinated with the killed vaccine *vs.* those receiving placebo was not significant (one-sided Fisher's exact test,  $P = 0.47$ ).

sided Fisher's exact test,  $P < 0.001$ ) *vs.* those without an antibody response.

In groups 1 and 2, 44% (4/9) and 60% (6/10) of the crows survived, respectively, resulting in an overall vaccine efficacy of 53% for parenterally administered DNA formulations. Statistically, the difference in survival between the crows that received the DNA vaccine parenterally and those receiving placebo was significant (one-sided Fisher's exact test,  $P < 0.0046$ ). In group 4, 11% (1/9) of birds vaccinated with the killed vaccine survived; this was not statistically different than survival of the unvaccinated controls (one-sided Fisher's exact test,  $P = 0.47$ ). None of the birds in groups 3, 5, or 6 survived WNV challenge (Table 1).

Mean peak viremia titers in the surviving crows ( $10^{4.2}$  PFU/ml) was significantly less than in the birds that died ( $10^{10.5}$  PFU/ml; one-way ANOVA,  $P \leq 0.001$ ) (Table 2). Vaccine history had no effect on mean viremia titers for the birds that died following WNV challenge. Likewise, the mean viremia titers of the birds that survived were similar, regardless of vaccine history. Not only were viremia

levels lower in surviving birds, but the mean number of days during which crows had viremias of  $\geq 10^5$  PFU/ml was lower in surviving birds as compared to those in birds that died (1.1 *vs.* 3.8 days, respectively). Further, viremia levels of  $\geq 10^7$  PFU/ml were observed for a mean of 0.1 days in crows that survived, *vs.* 3.2 days in crows that died.

## DISCUSSION

Because it is a highly susceptible avian species to New World strains of WNV in North America (18), the American crow represents a useful model for WNV vaccination. Likely vaccine candidates for WNV include valuable exotic, zoo, or endangered birds, although there is also prospective use for widespread vaccination of free-ranging birds when logistically possible (e.g., oral administration is efficacious, and relatively large numbers of birds can be reliably and safely exposed to the vaccine). In our study,

Table 2. Comparison of mean peak viremia levels in American crows that survived or died after challenge with virulent WNV.<sup>A</sup>

Group	Vaccine	Number tested	Survivors			Nonsurvivors		
			Number survived	Days viremia $\geq 10^5$ PFU/ml <sup>B</sup>	Peak viremia (range) <sup>C</sup>	Number died	Days viremia $\geq 10^5$ PFU/ml <sup>B</sup>	Peak viremia (range) <sup>C</sup>
1	DNA (i.m.)	9	4	0.5	3.9 (<1.7–6.5)	5	3.6	10.6 (10.3–10.8)
2	DNA (i.m.) + adjuvant	10	6	1.7	4.8 (<1.7–7.9)	4	4.8	11.0 (9.7–11.6)
3	DNA (oral)	10	0	NA	NA	10	3.5	10.2 (8.7–10.8)
4	Killed (i.m.)	9	1	0	<4.7 (<4.7) <sup>D</sup>	8	3.9	10.6 (8.5–11.3)
5	Adjuvant (i.m.)	8	0	NA	NA	8	4.5	10.3 (8.3–11.4)
6	PBS (i.m.)	10	0	NA	NA	10	4.0	10.5 (9.8–10.9)

<sup>A</sup>PFU = plaque forming units; i.m. = intramuscular; NA = not applicable; PBS = phosphate-buffered saline.

<sup>B</sup>Mean number of days that each crow had a viremia of  $\geq 10^5$  PFU/ml of serum.

<sup>C</sup>Log<sub>10</sub> geometric mean peak viremia levels. The detection threshold was  $10^{1.7}$  PFU/ml of serum, and a value of 1.7 was assigned to birds with undetectable viremia levels for the calculation of the mean. The mean peak viremia titer in surviving crows ( $10^{4.2}$  PFU/ml) was significantly less than in those that died ( $10^{10.5}$  PFU/ml; one-way ANOVA  $P \leq 0.001$ ).

<sup>D</sup>One crow with undetectable viremia levels was only able to be tested at a serum dilution of  $\geq 1:1000$ .



parenteral administration of a WNV DNA vaccine resulted in a weak humoral immune response in American crows, which is consistent with several other studies examining use of this vaccine in birds (7,26). When crows were challenged with virulent WNV, the vaccine-induced antibody response provided significant protection from mortality. However, even in surviving crows, sterile immunity was not always achieved. The addition of adjuvant did not significantly affect the performance of the DNA vaccine.

When compared to the DNA vaccine, the killed WNV vaccine did not elicit or maintain a sufficient antibody response or improved survival rate to warrant use of this vaccine in crows. Less than half of the birds in the killed-vaccine group developed a detectable antibody response, and survival of vaccinated birds after WNV challenge was not statistically different than those in the placebo group. On the other hand, the DNA vaccine (both with and without adjuvant) elicited a significant antibody response in 79% of the birds, and significantly increased the overall survival rate as compared to the control group (53% *vs.* 0%, respectively). Increased survival was associated with the presence of detectable antibody at either 6 or 9 wk after vaccination. This vaccine is not yet commercially available but shows promise for use in birds and warrants further studies.

Our results have implications on WNV transmission dynamics, demonstrating that vaccination in birds has the potential to decrease transmission among vertebrates and mosquitoes. In 60% of the surviving crows, the mean peak viremia was briefly elevated above the infectious level needed to infect a feeding *Culex pipiens* or *Culex quinquefasciatus* mosquito, which is approximately  $10^5$  PFU/ml of blood (27). Therefore, the vaccine did not reduce viremias in surviving crows to levels where transmission of the virus would be completely interrupted. However, reducing the mean peak viremia from about  $10^{10}$  to just over  $10^5$  would greatly reduce the percentage of mosquitoes feeding on these crows that would become infected. For example, about 80% of *Cx. pipiens* (one of the principal vectors of WNV in North America) became infected when feeding on a bird with a viremia of  $>10^7$  PFU/ml, whereas  $<5\%$  became infected when feeding on birds with viremias of about  $10^5$  PFU/ml (27). For crows that died because of WNV infection in our study, each bird had a viremia of  $\geq 10^7$  PFU/ml for an average of 3.2 days. In comparison, only one of 10 crows that received parenteral DNA vaccine and survived challenge had a viremia of  $\geq 10^7$  PFU/ml (for 1 day's duration), resulting in a group average of 0.1 day with a viremia of  $\geq 10^7$  PFU/ml. Therefore, the potential for these vaccinated birds to serve as a source of infection to mosquito vectors is greatly reduced. Other important WNV-amplifying hosts, such as the house sparrow (*Passer domesticus*), develop lower peak viremia titers than crows, and use of the DNA vaccine in these avian species might result in a more complete interruption of transmission to mosquitoes.

In the current formulation, the WNV DNA vaccine could be administered i.m. to protect valuable or endangered birds. Neither the killed vaccine nor the oral formulation of the DNA vaccine produced a significant immune response or decrease in mortality. Mean peak viremia levels following challenge of birds in the killed and oral DNA vaccine groups were comparable to those observed in the control groups. Failure of the oral vaccine is enigmatic, but could be because of DNA inactivation within the avian gastrointestinal tract or induction of nonneutralizing, nonprotective immunity. Similarly, the inability of the killed vaccine to protect crows, in contrast to its efficacy in horses, could be related to accelerated rates of WNV replication within corvid tissues (18). A strong humoral immune response is required for protection against WNV challenge. The ability of the DNA vaccine to elicit cellular expression of WNV

proteins similarly to their expression following natural infection may have led to a more effective immune response than occurred with viral antigen alone.

This work explored novel methods for controlling WNV infection in the American crow. The formulations used in this study failed to achieve complete protection. Additional studies will need to be undertaken as alternative WNV vaccines become available. The development of an oral WNV vaccine for use in wild birds has tremendous potential for reducing the risk of WNV exposure to humans and wildlife, as well as protecting numerous species of free-ranging and domestic animals.

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